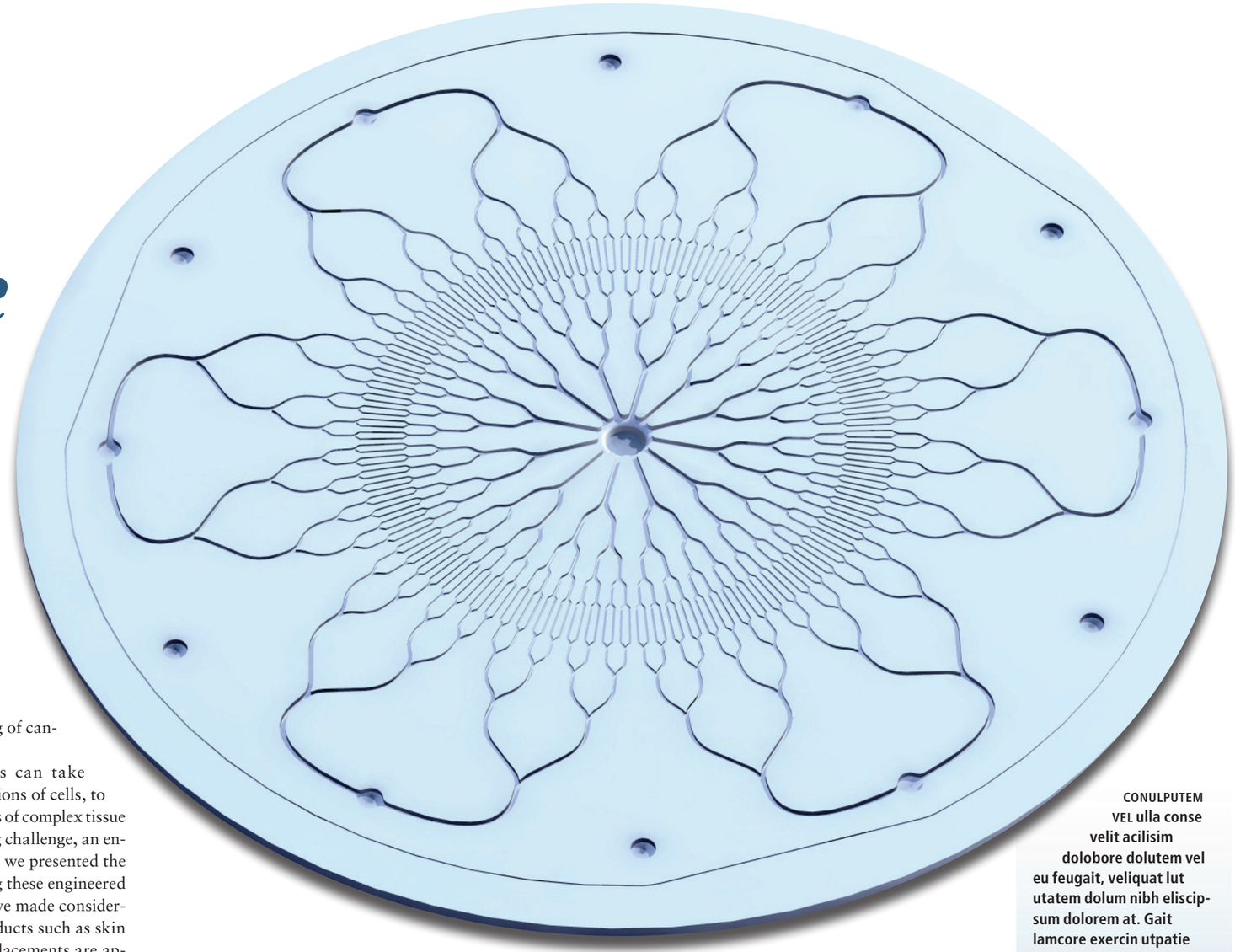
Progress in Tissue ENGINEERING

Pioneers in building living tissue report important advances over the past decade

By Ali Khademhosseini, Joseph Vacanti, and Robert Langer



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—The Editors

When two of us (Langer and Vacanti) last wrote in this magazine about scientific, ethical and business aspects of tissue engineering, the very idea that living flesh could be "constructed" using engineering principles and materials together with cells sounded fantastical to many encountering it for the first time. Yet the need for such transplantable human tissues to replace, restore or enhance organ function was, and remains, urgent. Today, nearly fifty million people in the United States are alive because of various forms of artificial organ therapy and one in every five people over the age of 65 in developed nations is likely to benefit from organ replacement technology during the remainder of their lives.

Current organ-substitution technologies, such as whole-organ transplants and dialysis machines, have saved many lives but they are imperfect solutions that come with heavy burdens for patients. Engineered biological tissues therefore have the potential to make a significant positive impact on the lives of people with failing organs, and to fill other human needs as well, for example, serving as "organs on a chip" for toxicity testing of candidate drugs.

Engineered tissues can take many forms – from aggregations of cells, to thin sheets or thick constructs of complex tissue and, the ultimate engineering challenge, an entire functioning organ. Since we presented the obstacles involved in creating these engineered tissues in 1999, scientists have made considerable progress and today products such as skin substitutes and cartilage replacements are approved for clinical use and have benefited thousands of patients. Artificial tissues such as bladder, bone, cornea and blood vessels are in clinical trials. And laboratory work on building more complicated tissue structures is also producing encouraging results.

Although some of the obstacles we described 10 years ago remain, significant advances over the past decade have come from new insights into the way that the body naturally builds tissues, both during embryonic development and natural wound healing. Engineering approaches toward assembling tissue structures have become more sophisticated, as have the chemical, biological and mechanical properties of the materials available to tissue engineers. For these reasons, regulatory agencies such as the U.S. Food and Drug Administration (FDA) are better able to assess the risks and benefits of tissue engineered products and the field is coming of age.

Delivering Life's Blood

One of the reasons that tissues such as skin, bladder and bone were first to be ready for testing in patients is these tissues do not require an internal vasculature. Cells are busy factories, manufacturing proteins and chemicals, and their constant activity quickly depletes the oxygen and nutrients in their surroundings. When deprived of this fuel, they can become irreparably damaged, therefore a constant supply of fresh blood is essential to keeping alive any tissue greater than a few hundred micrometers thick. The need to incorporate functioning vasculature into most engineered tissues has always been a significant challenge and a limitation on how large such constructs could become.

In the past few years, however, a number of new approaches toward building blood vessels – both outside tissues and within them – have

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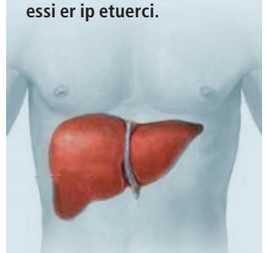

[BACKGROUND]

WHAT ARE SCIENTISTS TRYING TO DUPLICATE?

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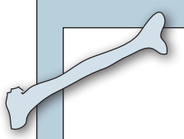

BONE: ILLO OF MICROSTRUCTURE

SKIN OR BLADDER: ILLO OF MICROSTRUCTURE

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Liver illustration with insets to show the layers of different cell types, neural connections, blood vessels, etc.

These are all things that tissue engineers need to reproduce.

been developed. Many of them rely on an improved understanding of the environmental needs of the endothelial cells that line blood vessel walls as well as the ability to sculpt materials at extremely small scales. For example, creating a bed of scaffolding material whose surface is patterned with nanoscale grooves – one thousanth the diameter of a human hair –encourages endothelial cells to form a network of capillary tubes. The grooves more effectively mimic the texture of body tissues that endothelial cells might rest against while forming natural blood vessels than a smooth surface would, providing an important environmental signal.

Microfabrication, the set of techniques used to make microelectronics chips for computers and mobile phones, has also been used to make capillary networks. For instance, one of us (Vacanti), with colleagues, has generated arrays of microchannels that mimic tissue capillary networks directly within degradable polymer scaffolds. Inside these channels, endothelial cells can be cultured to minimize the fouling effect of blood on the scaffold materials. Functional cells, such as liver hepatocytes, can also be separated from these capillary networks by using a membrane filter that allows for the exchange of nutrients and oxygen from the channels lined with the endothelial cells.

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Another method for keeping cells and blood separate but close enough to exchange a variety of molecules is to suspend them within hydrogels, which are jello-like materials made from hydrated networks of polymers. Since hydrogels chemically resemble the natural tissue matrix that surrounds all cells, the cells can be encapsulated inside the material, while channels through the gel can be coated with endothelial cells to engineer tissue-like structures with a protovasculature.

Research from the laboratories of Laura Niklason of Yale University and one of us (Langer) has shown that larger blood vessels can be generated by exposing vascular scaffolds seeded with smooth muscle cells and endothelial cells to pulsatile conditions inside a bioreactor. Arteries made in this environment designed to simulate conditions inside the body are mechanically robust and remain functional after transplantation into animals. In addition to enabling various tissue engineering applications, such engineered vessels by themselves may provide grafts for bypass surgery in patients with atherosclerotic disease.

Although the ability to engineer capillary-like structures and larger blood vessels outside the body is a significant breakthrough, a functional engineered tissue implant will have to connect quickly with the recipient's own blood supply to keep the construct alive. Coaxing the body to form new vasculature is therefore an equally important aspect of tissue engineering work. David Mooney of Harvard University, for example, has demonstrated that controlled release of growth factors from polymeric beads or from scaffold material itself can promote the formation of blood vessels that penetrate implanted tissue constructs. By culturing different cell types, such as smooth muscle cells and endothelial cells, together prior to implanting a tissue engineered construct, the formation of blood vessels and their integration into the host vasculature can also be enhanced. In fact, Pervasis Therapeutics Inc., with which two of us (Langer and Vacanti) are affiliated, is conducting advanced clinical trials in which a three-dimensional scaffold containing blood vessel cells is transplanted adjacent to the site of a vascular injury to provide growth stimulation and promote natural healing.

Despite these advances, a number of challenges still remain in making large vascularized tissues and vascular grafts and scientists have not yet completely solved this problem. New blood vessels grow and penetrate an implanted tissue construct slowly, causing many of the construct's cells to die for lack of a blood supply immediately after implantation. For this reason, tissue engineering approaches that include a vascular system pre-fabricated within the tissue construct are likely to be beneficial for large transplants. Such prefabricated vessels may also be combined with controlled release of blood vessel-recruiting growth factors to induce further formation and remodeling of the construct's vessels.

Integrating the engineered vasculature into the host vasculature is also very important, and researchers need a better understanding of the cross-talk between the host tissue cells and implanted cells to foster their connection. This need to decipher more of the signals cells exchange with one another and with their environments also extends to other aspects of building a successful tissue implant, such as selecting the best biological raw materials.

ALREADY IN HUMAN USE

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Suitable Cells

In most situations, building an implantable tissue from a patient's own cells would be ideal because they are compatible with that person's immune system. Realistically, such implants might also face fewer regulatory hurdles because the material is derived from the patient's own body. The ability of normal body cells to multiply in culture is limited, however, making it difficult to generate sufficient tissue for an implant. So-called adult stem cells from the patient's body or from a donor are somewhat more prolific, and they can be isolated from many sources, including blood, bone, muscle, blood vessels, skin, hair follicles, intestine, brain and liver.

Adult stem cells are difficult to identify, however, because their appearance is no different from regular cells in the same tissue, so scientists use the presence of distinctive surface proteins as molecular markers to flag stem cells. Further research to identify and validate more such markers would make it considerably easier to work with adult stem cells in tissue engineering applications. Over the past few years a number of major advances have been made in the field of adult stem cells, many of which involve novel methods of isolating them, inducing them to expand in number and to differentiate into various tissue types in culture.

Christopher Chen and Dennis Discher at the University of Pennsylvania have demonstrated, for example, that mesenchymal stem cells, which are typically derived from muscle, bone or fat, will respond to surrounding mechanical cues such as TK by differentiating into TK. Other researchers have also shown that chemical signals are important for directing the differentiation of adult stem cells into one tissue type or another [TK-need example]. Scientists disagree, though, about whether adult stem cells are able to give rise to cells outside their own tissue family – for instance, whether a mesenchymal stem cell could generate nerve cells.

In contrast to the some of the difficulties involved in working with adult stem cells, embryonic stem (ES) cells are easy to expand in culture and can differentiate into all the cell types of the

human body. One of us (Langer) along with Shulamit Levenberg of TKinstitution and colleagues, has demonstrated that ES cells can even be made to differentiate into a desired tissue type right on tissue engineering scaffolds. This capability suggests the potential to make 3D tissues on scaffolds directly from differentiating ES cells. These cells present a number of challenges, however.

Uniformly directing the differentiation of ES cells into the desired cell types is still quite difficult. In attempts to mimic the ES cells' complex natural microenvironment and optimize their differentiation, many conditions have been tested simultaneously to find the right combination of cues from a variety of materials and matrix molecules. Furthermore, researchers are screening various small molecules as well as signaling proteins to identify factors that direct both stem cell differentiation and the ES cells' ability to give rise to progeny cells while preserving their own "stemness."

Those insights could also be applied to producing cells with the capabilities of embryonic cells but fewer of the drawbacks. A major challenge for stem cell therapy is the inability to predict the behavior of transplanted stem cells in patients. Undifferentiated ES cells can form tumors, for instance, creating a risk of cancer if the cells are not all successfully differentiated before transplantation. In addition, efforts to address the ethical issues associated with deriving ES cells from human embryos have researchers exploring alternative approaches to creating ES-like cells from nonembryonic sources.

In the past couple of years, remarkable progress has been made in producing ES-like cells from regular adult body tissue, such as skin cells. These altered cells, known as induced pluripotent stem cells (iPS), are emerging as an exciting alternative to ES cells as a renewable resource for tissue engineering. Shiro Yamanaka of TK institution first showed that cells of adult tissue can be transformed to a primitive iPS state by reactivating a number of genetic pathways that are associated with stemness.

Reintroducing as few as four master regulatory genes into adult skin cells, for instance, caused the cells to revert to a primitive embryonic cell type. The early experiments inserted those genes into the cells by using a virus to transport them, a technique that would be too dangerous to use for tissues destined for patients. However, numerous researchers have followed on the original work by demonstrating different techniques for activating the same repertoire of stemness genes in the cells without the need for viral carriers. The rapid progress in this area has tissue engineers hopeful that soon a patient's own cells, endowed with ES cell capabilities, could become the ideal material for building tissue constructs. And even as we experiment with these different cell types, tissue engineers are also refining our building methods.

[THE AUTHORS]

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FDA REGULATIONS

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Architectural Advances

A decade ago, researchers assumed that cells are smart, and if we put the correct cell types in close proximity to one another, they would "figure out" what to do and form their native tissues. To some degree, this approach is effective but we now have a greater appreciation of the intricacy of signaling exchanged among different cells and their environments during organ and tissue development as well as during normal functioning.

Each tissue in the body performs specific tasks that must be recreated within tissue engineered constructs, and we are learning that replicating the underlying biology of the tissue in question as closely as possible is critical to generating tissues that can perform their intended functions. One dramatic example mentioned above is the robustness of vascular or heart muscle tissue when it is grown in conditions that replicate the pulsing of blood through the body. In more complex organs, multiple cell types work in concert – in the liver, for instance, the cells have many tasks including detoxification and nutrient breakdown. Therefore, the microarchitecture of tissues and the positioning of cells relative to other cells must be recreated in tissue-engineered constructs in order to recreate the desired functionality.

In most natural tissues, the surrounding cellular microenvironment is controlled with a fine degree of spatial and temporal organization and early tissue engineering work used scaffolds made from various materials to try to replicate the 3-D shape of the tissue as well as crudely approximating this cell organization. A number of advances have been made in the past few years to enhance the level of complexity of engineered tissues and to reproduce the tissue environment more closely. For example, scaffolds made from actual natural tissues that have had all cells removed, leaving only connecting fibers, have been used to grow engineered tissues that recreate a significant amount of the function of the original tissue. In one study, decellularized rodent heart scaffolds that were seeded with cardiac and endothelial cells produced cardiac muscle fibers and vascular structures that formed a beating heart.

Assorted "printing" technologies have also been used to generate tissues one cell at a time. By modifying standard inkjet printers, cells themselves or scaffold materials can be dispensed with precision to generate tissues or frameworks with controlled spatial organization onto which cells can be seeded. Mimicking the tissue's natural topography also helps to guide the cells, and microfabrication of surfaces is one method of controlling the scaffold surface to a fine degree for growing blood vessels or other tissues.

Another technology borrowed from the engineering world, electrospinning, can also produce scaffolds with features that resemble natural tissue matrix topography. Very thin polymer fibers are spun to form weblike scaffolds, which provide cells with a more natural 3D environment, and the chemical and mechanical features of the polymer materials can be finely manipulated. David Kaplan of Tufts University has made similar scaffolds from silk materials that resemble spider webs to generate ligaments and bone tissues.

Because the biological, chemical and mechanical properties of hydrogels can be significantly tailored, the gels are also proving to be useful materials for supporting and encasing cells while enhancing the function of the resulting tissues. Hydrogels containing live cells can be "printed" or otherwise arranged and layered to delineate correct tissue structure. One of us (Khademhosseini) has shown, for example that hydrogel-encased cell aggregates can be molded into any number of complementary shapes (see illustration on page 00), then pooled together to self-organize into a larger complex pattern. This technique could be used to replicate the natural organization of cells in a tissue such as the liver, which is made up of hexagonal lobes that each contain toxin-filtering cells surrounding a central blood vessel.

Some gels are designed so that their polymers link together in response to UV light, making it possible to create the desired construct shape and then make all or parts of the construct firm by exposing them to light. Kristi Anseth of the University of Colorado and Jennifer Elisseeff of Johns Hopkins University have demonstrated

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Advances in Encouraging Vascular Growth

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CASE STUDY: NANOSCALE GROOVES

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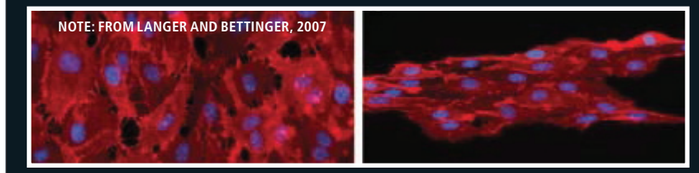

CASE STUDY: MICROFABRICATION

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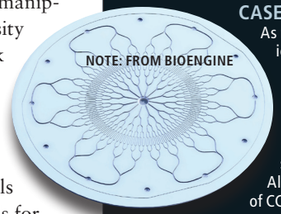

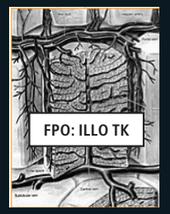

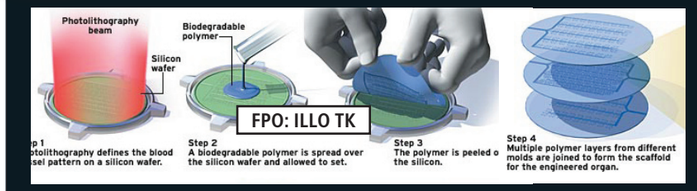

CASE STUDY: COAXING THE BODY TO FORM NEW VASCULATURE

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MICROGRAPH OR ILLUSTRATION BASED ON WORK IN WHICH 3-D SCAFFOLDS CONTAINING BLOOD VESSEL CELLS ARE TRANSPLANTED ADJACENT TO SITE OF VASCULAR INJURY

SOMETHING FROM PERVASIS THERAPEUTICS?

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Advances in Building Microarchitecture

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CASE STUDY: PRINTING TECHNOLOGIES

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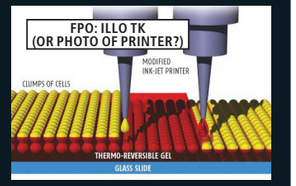

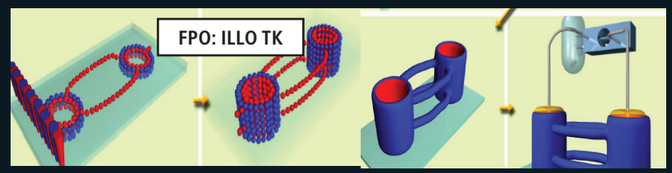

MICROGRAPH?

FROM ONE OF OUR AUTHORS, OR KAPLAN OF TUFTS

CASE STUDY: ELECTROSPINNING

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CASE STUDY: LIVING LEGOS

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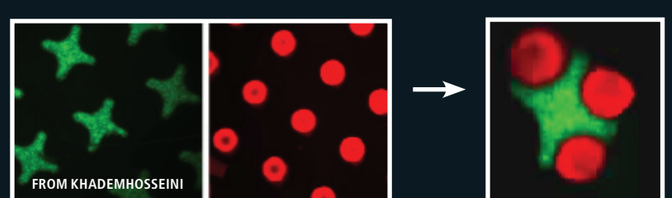

FROM KHADEMHOSSEINI

the use of photocrosslinkable hydrogels for cartilage and bone tissue engineering, for example. Gels can also be imbued with a number of signaling molecules to promote tissue growth or differentiation. For instance, Samuel Stupp of Northwestern University has shown that neural stem cells will differentiate into neurons within a hydrogel that incorporates small proteins that act as environmental signals directing the cells' behavior.

Finally, nanotechnology has also been used to generate engineered sheets of cells that can be used for transplantation. Teruo Okano of Tokyo Women's Medical University has generated surfaces coated with a temperature-responsive polymer that swells as the temperature is lowered from 37 °C to 20 °C. On these surfaces cells are induced to form a single layer. Subsequently, the temperature is lowered to swell the underlying substrate and detach the intact cell sheet These cell sheets, which contain appropriate cell-secreted matrix molecules can then be stacked or rolled for generating larger tissue constructs.

While these advances have made a significant improvement in the range and diversity of scaffolds that can be generated, challenges remain in this area as well. One difficulty is the lack of knowledge of the concentrations and combinations of growth factors and extracellular molecules that are present at specific stages of development and wound healing in various tissues. A better understanding of these design parameters is needed to engineer tissues that mimic the body's own healing and development. Thus, tissue engineers are looking to other fields, including studies of gene and protein interactions in developing tissues and regenerating wounds, and combining these insights with advanced culture systems to better control the response of cells outside the body. Greater sophistication in scaffold materials is allowing tissue engineers to better recreate the spatial and temporal aspects of the cells' microenvironments, but further research and development in this area is required.

Coming of Age

Despite the ongoing challenges we have described, engineered tissues are no longer a fantastical prospect. Simple manufactured tissues are already in clinical use, and this method of restoring or replacing biological function is now poised to become a viable therapy for millions of patients in need. As of mid-2007, more than 50 companies had already commercialized various tissue engineering products with annual sales in excess of $1.3 billion.

Those figures are all the more impressive in light of setbacks to the field that occurred shortly after last we wrote for this magazine about tissue engineering's promise. At the end of the 1990s and in the early 2000's, enthusiasm and investment were high, but with the burst of the internet financial bubble, funding for biotechnology start-ups dwindled. Even companies with FDA-approved tissue engineered products had to restructure their business models, delaying introduction of their products to the market.

Because engineered tissues are made from cells, biologically active chemicals and nonbiological scaffold materials, the constructs must undergo rigorous analysis by the FDA, which is costly and time-consuming. A lack of funding made conducting extensive clinical trials more difficult for companies, but the delay in commercializing some tissue engineered products bought time for the science to mature and business approaches to become more sophisticated.

And there is still room for improvement. One difficulty in bringing engineered tissues to the clinic is the inherent variability of cells from different sources and of individual recipients. Variations in tissue healing and remodeling responses create an unpredictability that presents a challenge for FDA approval. Further research to measure and understand such variations between individuals and to account for them in clinical trials that study tissue engineered products is therefore important. And future business models must account for the extensive costs that will be associated with this work as well.

The advances we have described in the materials available and in the biological understanding of engineered tissues have nonetheless enabled the field to bounce back from past downturns and made it possible to engineer tissue constructs with more functional capability than ever before. With a new appreciation for the importance of developmental and regenerative biology, tissue engineers are now aiming to create second-generation tissues that are more mechanically, chemically and functionally close to their biological counterparts.

We believe that the next few years will bring significant developments in the merger of stem cell biology, nanotechnology and an understanding of the biology of cell systems with tissue engineering. This combined approach to generating artificial organ substitutes may finally provide a suitable solution to the numerous medical and biotechnology challenges that require engineered biological tissues.

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COMPANY/PRODUCT TABLE

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